

APPENDIX T

FILTRATION AND ELUTION OF SAMPLES BY U.S. ENVIRONMENTAL PROTECTION AGENCY METHOD 1623 – ULTRAFILTRATION METHOD

Ultrafiltration Method for Concentration of *Cryptosporidium* oocysts and *Giardia* cysts in Water

EQUIPMENT

1. Filter (Fresenius Hemoflow F80A Capillary Dialyzer)
2. Tubing (silicone; Masterflex size 17, 3/8"ID)
3. Cubitainer (10 L), sterile stir bar, and spigot
4. Stir plate
5. Ring stand and clamp
6. Filtrate catch basin (e.g., bucket)
7. Peristaltic pump with easy load L/S Precision pump head that fits size 17 tubing
8. pressure valve
9. plastic tees for 3/8" tubing
10. swaglok fittings – stainless steel
11. luer locks
12. (add plumbing supplies)

REAGENTS AND STANDARDS

1. Laureth-12
 - a. BASF Corp (#558208)
2. Dulbecco's phosphate buffered saline (PBS)
 - a. Sigma (D-5652)
3. Antifoam A
 - a. Sigma (A-5758)
4. Tween 80
 - a. Sigma (P-5188)
5. SigmaCote
 - a. Sigma (SL-2)
6. Elution Solution (1X PBS / 1% Laureth-12 solution).
 - a. Add 9.55 g of PBS to 800 mL deionized water in a 1-L Wheaton bottle. Shake until dissolved.
 - b. Weigh 10 g of Laureth-12 into 500-mL beaker, being sure to not smear onto sides of beaker.
 - c. Add approximately 100 mL of PBS solution to beaker.
 - d. Heat to dissolve Laureth-12
 - i. Do not use stir bar; swirl often
 - ii. Heat until solution becomes clear (no wavy smears)
 - iii. DO NOT BOIL
 - e. Pour this solution into new, 1-L Wheaton bottle. The bottle should be labeled with date made, and lot numbers of PBS and Laureth-12.
 - f. Use all of the remaining PBS solution (approx. 700 mL) to rinse beaker thoroughly several times.

- g. Add 3 drops of Antifoam A, then mix.
- h. Bring volume to 1 L with deionized water.
- i. Autoclave for 15 min.
- j. Store at 4°C; discard after 1 week.

METHODS

1. Pre-treat ultrafilters overnight with elution solution.
 - a. Elution solution (1X PBS/1% Laureth –12 solution).
 - b. Set up system as diagrammed in Figure 1.
 - i. Sterilize tubing by soaking in 10% hypochlorite solution for five minutes and then rinse thoroughly with tap water then DI water or use new tubing.
 - ii. For this setup only, the filtrate ports should be pointed upward to allow for collection of solution within the filter.
 - c. Circulate elution solution through the system (10-15 psi) until a small amount has collected at the bottom of the ultrafilter.
 - d. Allow the ultrafilters to soak overnight within sterile packaging or on the experimental setup.

2. Pre-treat 15mL centrifuge tubes for transport of final sample to analyzing laboratory.
 - a. Obtain several 15-mL sterile, polypropylene centrifuge tubes.
 - b. Pour SigmaCote into tube, cap, vortex, and shake tube. Use approximately 2 mL for each tube.
 - c. Pour out the SigmaCote either back into bottle or into next tube, turn upside down in a rack (uncapped) until dry.
 - d. Once dry, cap the tube loosely in a 2-L beaker, cover with foil, and autoclave for 15 minutes.

3. Paperwork
 - a. Log in sample.
 - i. Date received
 - ii. ODML login: “C01-xxxa” to “C01-xxxd”
 - iii. Site name
 - iv. Sample collection date and time
 - v. Sample type
 1. a – regular
 2. b – cell culture
 3. c – ColorSeed spike
 4. d – EPA spike
 - b. Label centrifuge tubes with sample ID#, date, initials, and sample collection site.
 - c. Enter chain of custody information (received by, and date received).
 - d. Label top of bench sheet with sample information.
 - e. Take pH, specific conductance, and two turbidity readings from 3-L bottle; **record results on bench sheet.** (This can also be done when sample is filtering).
 - f. If spiking the sample, fill out relevant information on bench sheet.

4. Ultrafiltration can be performed the next morning.
 - a. Re-create ultrafiltration system with pre-treated filter. Be sure filtrate hoses are in the filtrate catch.
 - b. Prior to attachment to setup, place sterile stir bar in sample carboy. Replace the cubitainer cap with a spigot. Stir sample 5 min prior to filtration. Keep sample stirring as long as possible. The sample should be elevated (approximately 25 cm above bench top).
 - c. **Record start time, name, and lot number of filter.**
 - d. Ultrafilter sample at 20-25 psi until the sample is reduced to the hold-up volume of the ultrafilter and tubing. This could take approximately 1 hour. Once running, the system can be left unsupervised for short periods of time. Checking should occur every fifteen minutes or less to be sure the sample is still filtering and there are no leaks. **Record end time and measure volume of filtrate.**
 - e. Discard filtrate in sink after measuring volume.
 - f. Rinse sides of cubitainer with sterile DI water and run through filter.
5. Concentrated sample/microbes should be eluted with elution solution.
 - a. Add 250 mL of elution solution to 250-mL centrifuge tube. **Record date made and lot numbers of Laureth 12 and PBS.**
 - b. Detach tubing from plastic tee and place in elution solution in centrifuge bottle.
 - c. Slowly circulate elution solution through ultrafilter (5-10 psi) until reduced to hold-up volume of ultrafilter and tubing. There should be about 5 mL in centrifuge tube.
 - d. Remove inlet hose from centrifuge bottle and allow liquid to fill bottle.
 - e. Gently use positive-pressure air to blow out any remaining liquid by attaching inlet to positive air source. Be sure tubing is clear. If necessary, lift tubing and use gravitational force as well as the air pressure (<25 psi) to push sample through system.
6. Further concentration of sample/microbes by centrifuge.
 - a. Settings for centrifuge
 - i. 4°C, allow to come to temperature prior to use.
 - ii. Accel = 9; Brake = 0; Rotor = 259
 - b. Place flat black pad in centrifuge bucket; add gray plastic cushion and then the centrifuge tube. Do not use lid on bucket.
 - c. Balance tubes by adding reagent water into centrifuge tube.
 - d. Centrifuge the 250-mL centrifuge tube at 1,500 x G for 15 minutes.
 - e. Allow centrifuge to stop.
 - f. Using a glass Pasteur pipette with vacuum, carefully aspirate the supernatant to the 5-mL mark or 3mL above pellet, whichever is greater. Use glass pipette attached to vacuum flask and vacuum source. Be careful not to aspirate too much fluid and to not disturb the packed pellet. This could lead to aspiration of oocysts or cysts.
 - g. **Record volume of packed pellet and volume aspirated.**

7. Transfer of pellet.
 - a. Vortex the tube or use a pipet to resuspend the pellet.
 - i. Do not vortex sandy samples; this may cause breakdown of oocysts and/or cysts.
 - ii. When using pipet, do not stir; pull supernatant up into pipet and then release solution back into the tube to gently disturb the pellet so that it may be resuspended. Repeat several times.
 - b. Transfer entire volume to 15-mL sterile polypropylene centrifuge tube, which has been pre-treated with SigmaCote (as described above).
 - i. Pipet up resuspended pellet using a disposable, sterile pipet and release into the treated 15-mL centrifuge tube.
 - ii. Rinse larger centrifuge tube using two washes of no more than 2.5 mL each of DI water. If pellet volume and supernatant exceed 5 mL, use fewer mL for rinsing. Volume shipped should not exceed 10 mL!
 - iii. **Record total volume shipped.**

SPIKING FOR QUALITY CONTROL

1. Two spikes will occur with every sample.
 - a. ColorSeed, sterilized with gamma irradiation, and
 - b. Flow-counted live oocysts and cysts produced by the EPA.
2. Procedure for both spike suspensions.
 - a. Add 2 mL of 0.05% Tween 80 to the spike solution tube.
 - b. Replace the cap and shake vigorously; DO NOT vortex.
 - c. Remove cap and pour spike suspension into the sample.
 - d. Add 3 mL of reagent water to the tube.
 - e. Replace cap and shake vigorously.
 - f. Remove cap and pour the wash into the sample.
 - g. Repeat steps d-f twice more.
3. Safety
 - a. Avoid exposure to spike suspension.
 - b. Wear at all times gloves and lab coats.
 - c. Clean up any spills with 10% hypochlorite solution.
 - d. Autoclave all equipment that comes into contact with oocysts and cysts.

PROCEDURE FOR IPR/OPR

IPR's are done at the beginning of the project to establish proficiency. OPR's are done quarterly thereafter.

1. Use a clean and sterile 3-L bottle, cap, and nozzle to simulate field conditions.
2. Rinse the 3-L bottle and cubitainer with DI water.
3. Fill the 3-L bottle with DI water. Screw on the cap and nozzle. Empty the 3-L bottle into the cubitainer through the nozzle.
4. Repeat step 3 until the cubitainer is filled.
5. Continue filtration procedure as for regular sample.

SHIPPING

A cooler is sent to UNC in a box labeled as “infectious materials” with a dangerous goods shipping form. The following are included:

1. Sample tubes
2. 8 vials of spike
3. 2 vials of ColorSeed
4. Fedx form from UNC-EPA
5. ColorSeed certificate (copy)
6. Bench sheets (copy)
7. Service request forms (copy)

A small cooler is sent to USEPA with following:

1. 1 vial of spike
2. Return label to USGS